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A Mouse/Human-Chimeric Bispecific Antibody Reactive with Human Carcinoembryonic Antigen-Expressing Cells and Human T-Lymphocytes

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Abstract. *A mouse/human-chimeric bispecific antibody, designated CBA-CEACD3, with dual specificities for carcinoembryonic antigen (CEA) and CD3, was generated by chemical cross-linking of a chimeric antibody specific for CEA to another chimeric antibody against CD3. Flow cytometric analysis showed that CBA-CEACD3 can bind specifically to cells expressing CEA and to normal human peripheral blood mononuclear cells (HPBMCs) bearing CD3, respectively. Furthermore, a cell to cell adhesion analysis by a colorimetric assay using the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) demonstrated that CBA-CEACD3 is able to bind CEA-producing cells to CD3-expressing cells, suggesting that both arms of CBA-CEACD3 are simultaneously working and can retarget T-cells to the tumor. In an additional colorimetric assay using MTT, this antibody was shown to effectively mediate CEA-expressing tumor cell killing by freshly isolated HPBMCs. Together these results demonstrate that this chimeric bispecific antibody may serve as a potentially useful immunotherapeutic reagent for human CEA-producing cancers.*

Carcinoembryonic antigen (CEA), a highly glycosylated glycoprotein with a molecular weight of 180,000, is the most widely used tumor marker. Molecular biologic techniques have recently elucidated much biochemical information on CEA, *e.g.* its domain structure (1), structural relationships to other CEA-related antigens (2) and functional roles through adhesion activity (3). Furthermore, several studies have demonstrated that CEA is normally produced by colonic epithelial cells as actively as in cancerous tissues (4). In normal adult human colon tissues, however, CEA is localized on the luminal surface of the single layer of columnar epithelial cells lining the upper parts of the crypts, so that normal CEA is not directly facing blood flow or tissue fluid (5). On the other hand, in tumor tissues which no longer conform to the single-cell layer organization by invading through the basement membrane in multicellular arrays, CEA is usually localized at all sides of cell membranes and is directly facing blood flow or tissue fluid (3). Hence, tumor CEA is useful as the targeted molecule of immunotherapy using anti-CEA antibodies (5).

Hybridoma technology has yielded monoclonal antibodies (MAbs) with specificities to a wide range of antigens,such as tumor-associated antigens and lymphocyte cell surface markers. Subsequently, bispecific MAbs with dual specificities for tumor-associated antigens and for surface markers on immune effector cells have been developed as new agents for immunotherapy (6,7). These bispecific MAbs are usually produced by the chemical cross-linking of two MAb molecules or by the fusion of two hybridomas secreting different MAbs (8), and have proved effective for retargeting effector cells to kill tumor cells both *in vitro* and *in vivo* (9, 10). However, most of them are of murine origin and are confronted with the problems of strong immunogenicity and rapid serum clearance in humans (11-13). In a recent

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development of gene manipulation, murine antibodies have been genetically modified by fusing murine variable regions to human constant regions (14-15). These mouse/human chimeric antibodies have already been used in clinical fields and been shown to be less immunogenic than their murine counterparts (16).

In a recent study, we generated two mouse/human chimeric antibodies to CEA, designated Ch F11-35 and Ch F11-39, and showed that both chimeric antibodies exhibited the same high specificity and affinity for CEA as those of their parental mouse MAbs, respectively (15). Additionally, in *in vivo* biodistribution studies by tissue counting we demonstrated that the two ^{125}I-labeled chimeric antibodies were specifically localized in the tumor tissues of athymic nude mice bearing human CEA-producing tumor xenografts (17). More recently, we developed a mouse/human chimeric antibody (Ch OKT3) to the human T cell surface marker CD3 [Arakawa, F., unpublished results] from an anti-CD3 MAb, OKT3 (18). The human CD3 on T cells consists of a minimum of five polypeptide chains noncovalently associated with the T cell receptor, and plays an important role in the process of T cell activation in response to antigen recognition by the T cell receptor (19). In the present study, we prepared a mouse/human-chimeric bispecific antibody that recognized CEA and CD3 by chemical cross-linking of Ch F11-39 to Ch OKT3. The resultant chimeric bispecific antibody was purified by gel filtration and characterized immunochemically.

Materials and Methods

Generation and purification of chimeric antibodies. The construction, expression, and immunochemical properties of the mouse/human chimeric antibody to CEA, designated Ch F11-39, have been described in detail elsewhere (15, 17). This chimeric antibody exhibited the same high specificity and affinity for CEA as that of its parental mouse MAb, F11-39 (20). Another mouse/human chimeric antibody to CD3, Ch OKT3, was generated from the parental mouse MAb OKT3 (American Type Culture Collection, Rockville, MD) as previously described for Ch F11-39 (15). Briefly, total RNA was isolated from OKT3 hybridoma by the acid guanidinium thiocyanate-phenol-chloroform extraction method. Thereafter, the variable region genes of heavy (V_H) and light (V_κ) chains of OKT3 were cloned from the total RNA using the reverse transcriptase-polymerase chain reaction method. The resulting V_H and V_κ genes of OKT3 and the constant region genes (γ1 and κ) of human heavy and light chain derived from a human plasma cell leukemia line (ARH77) were fused in pSV2gpt and pSV2neo plasmid vectors, respectively. The chimeric heavy and light chain expression plasmids were cotransfected into mouse non-Ig-producing hybridoma (Sp2/0) cells by electroporation. The two transfectomas were grown in an artificial capillary culture system using CELLMAX™ (Cellco, Germantown, MD) (21). The two chimeric antibodies were purified from the extracapillary space medium of the artificial capillary culture system. Partially purified Ch F11-39 and Ch OKT3 with 40% saturated ammonium sulfate were applied to CEA- or anti-human IgG-Sepharose 4B, respectively. The antibodies were further purified by gel filtration on a Superdex 200 column (16 × 600 mm) connected to a fast protein liquid chromatography system (Pharmacia Biotech, Uppsala, Sweden) using 0.01 M borate buffered saline (BBS), pH 8.0. Protein contents of purified antibodies were measured by the bicinchoninic acid method (22).

Preparation of chimeric bispecific antibody by chemical cross-linking. The purified Ch F11-39 and Ch OKT3 were cross-linked using *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Pierce, Rockford, IL) according to the manufacturer's protocol (23, 24). Briefly, a threefold molar excess of SPDP was added to 5 mg of each antibody in 0.1 M potassium phosphate, 0.1 M NaCl, pH 7.5 (coupling buffer), and the mixtures were incubated separately for 2 hours at room temperature. The Ch OKT3 solution was redialyzed against coupling buffer. The pH of the Ch F11-39 solution was lowered to pH 4.5 with 0.1 M sodium acetate, 0.1 M NaCl, pH 4.5, and bound 3-(2-pyridyldithio) propionate groups were reduced with dithiothreitol at a final concentration of 0.02 M. After 30 minutes at room temperature, the Ch F11-39 was passed through a Pharmacia PD10 column equilibrated with coupling buffer, and immediately added to the nonreduced Ch OKT3 solution, resulting in a total volume of about 3 ml. After 4 hours incubation at room temperature, 1 mg of iodoacetamide was added and the reaction mixtures then were dialyzed in BBS overnight. Cross-linked chimeric bispecific antibody was separated from monomeric antibodies on a Superdex 200 column and designated CBA-CEACD3.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in 4-12% polyacrylamide gels according to the method of Laemmli (25), under nonreducing conditions. Proteins in the gels were visualized by staining with Coomassie Brilliant Blue.

Cell culture. Chinese hamster ovary (CHO) dhfr$^+$ transformants transfected with the vector pdKCR-dhfr-full-length CEA cDNA were prepared as described previously (26). CHO dhfr$^+$ transfectoma cells have been shown to express CEA in membrane-bound form. A human CEA-expressing gastric carcinoma cell line, MKN-45, provided by the Japanese Cancer Research Resources Bank (Tokyo), was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. Cells were harvested for the following experiments by incubation for 5 minutes at 37°C with 2 mM EDTA in Dulbecco's phosphate buffered saline free from Ca^{2+} and Mg^{2+} (PBS).

Preparation of human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells (HPBMCs) were obtained from heparinized peripheral blood of healthy human donors as described previously (27). Isolation was done by density gradient centrifugation of diluted (1:1 PBS) blood on Lymphoprep (Nycomed, Oslo, Norway) at 400 *g* for 30 minutes. HPBMCs collected from the gradient interphase were washed repeatedly with PBS to remove platelets.

Flow cytometry. To determine antibody binding to the cells, flow cytometry was performed (28). Aliquots of 2 × 10^6 cells, washed with PBS, were incubated with the antibodies to be tested in PBS containing 1% normal goat serum for 1 hour at 4°C. Cells were washed with PBS containing 1% normal goat serum and incubated with fluorescein-conjugated goat F(ab')$_2$ anti-human IgG (Cappel Research Products, Durham, NC) for 30 minutes at 4°C. As a negative control, a human IgG1(κ) myeloma protein from the Binding Site (Birmingham, England) was included. After washing with PBS, the stained cells were resuspended in 1.0 ml FACS medium, filtrated through a nylon mesh, and analyzed using a FACScan (Becton Dickinson, San Jose, CA).

Reactivity with purified CEA. The reactivities of CBA-CEACD3 with purified CEA were estimated by a solid-phase enzyme immunoassay (SPEIA) using CEA immobilized on 96-well plates (Greiner GmbH, Frickenhausen, Germany) as described previously (29). A highly purified CEA preparation was prepared as described previously (30). To equalize the number of moles of antibody, the amount of purified antibodies tested were determined from their molecular weights (see below).

Antibody-mediated adhesion of CEA-expressing cells to CD3-bearing cells. For determining the bifunctional binding activity of CBA-CEACD3, a

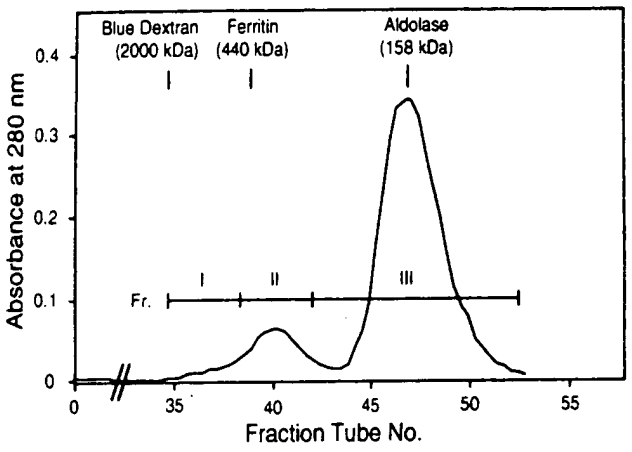

Figure 1. *Gel filtration profile of 1 ml of a reaction mixture prepared from Ch OKT3 and Ch F11-39 using SPDP on a Superdex 200 column (1.6 × 60.0 cm) operated with 0.01 M BBS at a flow rate of 0.5 ml/minute. The effluent was collected in 1.3 ml portions. Fraction II contained CBA-CEACD3 and was usedfor the experiments. Elution positions of markers were shown from the same column.*

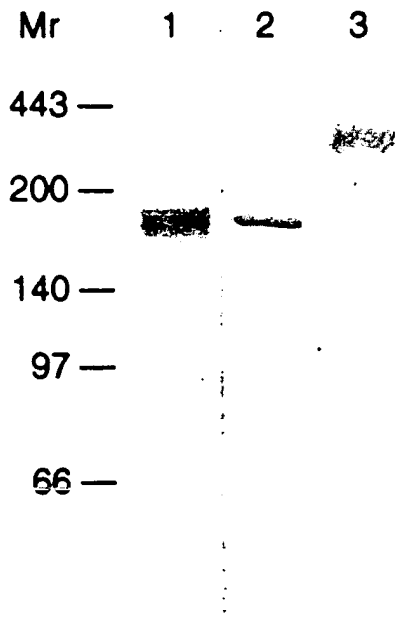

Figure 2. *SDS-PAGE analysis (4-12% polyacrylamide gel) of CBA-CEACD3 and its parental chimeric antibodies. About a hundred nanograms of each purified antibody were added to each lane. Lane 1, Ch OKT3; Lane 2. Ch F11-39; and Lane 3. CBA-CEACD3. Vertical scales, molecular weight markers (× 10-3).*

cell to cell adhesion assay using HPBMCs fixed on 96-well plates and free CHO dhfr+ transfectoma cells expressing CEA was performed. At first, 2 × 10^5 HPBMCs in 100 µl PBS were allowed to settle for 1 hour at 4°C to the bottom of each well of 96-well plates (Greiner) which had been treated with poly-L-lysine (20 µg/ml) for 30 minutes. The cells were fixed by incubation with 100 µl of 0.1% glutaraldehyde in PBS for 5 minutes at 4°C. The plates were blocked by incubation for 2 hours with 200 µl of Block Ace (Dainihon Chemical Industries, Osaka) which includes casein and some other proteins from bovine milk. CHO dhfr+ transfectoma cells were washed and suspended in serum-free and phenol red-free RPMI 1640 supplemented with 0.2% of bovine serum albumin. Fifty microliters of the cells were seeded in triplicate in the HPBMC-coated plates at a concentration of 5 × 10^6 cells/ml. Then, 50 µl of the antibodies to be tested were immediately added into each well. The number of moles of antibody was equalized as mentioned above. After centrifugation at 50 *g* for 5 minutes, the plates were incubated at 37°C in 5% CO_2 for 2 hours. Non-adherent cells were removed by carefully inverting, flicking and blotting the tray. Specifically binding cells were quantified by a colorimetric assay using the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Research Organics Inc., Cleveland, OH) as previously described (31). The percentage of cells bound was defined as the ratio of the mean value of absorbencies in experimental wells divided by the mean value of absorbence obtained after the same incubation time with total CHO dhfr+ transfectoma cells added in control wells.

Cytotoxicity assay. The efficacy of CBA-CEACD3 in directing tumor cell killing in the presence of HPBMCs was determined by a cytotoxicity assay using MKN-45 cells adhered to the well bottoms of 96-well plates. Briefly, 2.5 × 10^4 MKN-45 cells were first seeded into 96-well plates (Costar, Cambridge, MA). After 48 hours culture incubation, the plates were washed twice with PBS to remove non-adherent cells including dead cells. The effector cells at the indicated effector/target (E/T) cell ratios were added into each well of the plates in the presence of antibodies to be tested. The number of moles of antibody was equalized (5 nM each) as mentioned above. The plates were then incubated for 6 hours at 37°C, in 5% CO_2. The effector cells and killed tumor cells were removed by washing for 3 minutes under vigorous shaking on a plate shaker. The remaining living cells were immediately quantified by a colorimetric assay using MTT as mentioned above (31, 32). Cell killing was calculated according to the formula.

$$\% \text{ cell killing} = \frac{OD_{540} \text{ in the absence of antibody} - OD_{540} \text{ in the presence of antibody}}{OD_{540} \text{ in the absence of antibody}} \times 100(\%)$$

In a competitive inhibition assay, tumor cells were treated with CBA-CEACD3 in the presence of $F(ab')_2$ fragments of the parental mouse MAb OKT3 (100 nM) before addition of HPBMCs. The $F(ab')_2$ fragments of OKT3 were prepared as described previously (9).

Results

Preparation of CBA-CEACD3. Ch F11-39 specific for CEA and Ch OKT3 recognizing CD3 were chemically cross-linked using SPDP. Separation of the desired heteroconjugate from the reaction mixture was achieved by filtration on a Superdex 200 column. A typical elution profile is presented in Figure 1. Fractions from tubes 38 to 42 inclusive (49.4-54.6 ml) containing the molecules with a molecular weight of about 300 kDa were pooled for further experiments and designated

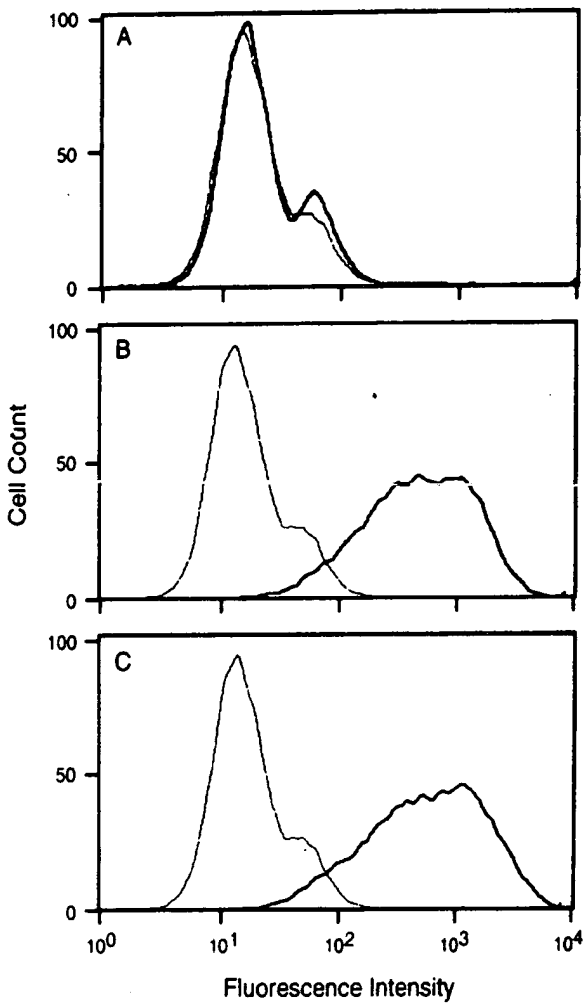

Figure 3. *Flow cytometlic analysis of the binding of CBA-CEACD3 to CHO dhfr+ transfectoma cells expressing CEA. Cells were incubated with Ch OKT3 (A), Ch F11-39 (B), and CBA-CEACD3 (C) before staining with fluorescein-conjugated goat F(ab')$_2$ anti-human IgG. Thin line shows the data obtained with control human IgG$_1$(κ) followed by staining with fluorescein-conjugated goat F(ab')$_2$ anti-human IgG.*

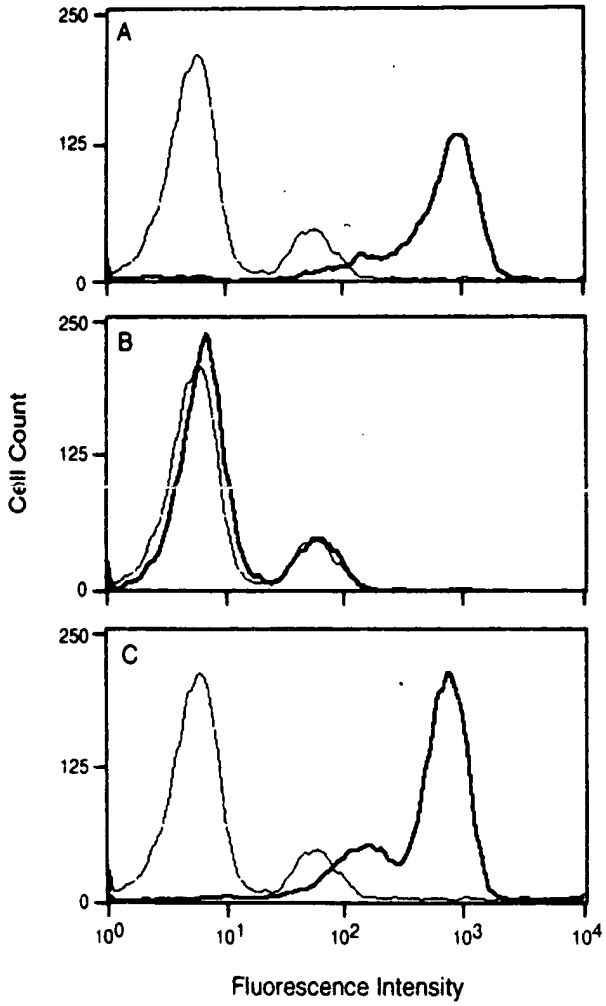

Figure 4. *Flow cytometric analysis of the binding of CBA-CEACD3 to HPBMCs bearing CD3. Cells were incubated with Ch OKT3 (A), Ch F11-39 (B), and CBA-CEACD3 (C) before staining with fluorescein-conjugated goat F(ab')$_2$ anti-human IgG. Thin line shows the data obtained with control human IgG$_1$(κ) followed by staining with fluorescein-conjugated goat F(ab')$_2$ anti-human IgG.*

CBA-CEACD3. When referred to the initial amount of the chimeric antibodies, an absolute yield of 15 % for CBA-CEACD3 was obtained.

Figure 2 shows the SDS-PAGE analysis under non-reducing conditions of CBA-CEACD3 and its parental chimeric antibodies (Ch F11-39 and Ch OKT3). Ch F11-39 exhibited a relatively clear band with a molecular weight of about 160 kDa, and Ch OKT3 showed a slightly broad band with a similar molecular weight of 160 kDa. Whereas CBA-CEACD3 showed a broader band and its molecular weight calculated for the center of the smear was about 300 kDa.

Cell binding reactivity of CBA-CEACD3 determined by flow cytometry. Binding reactivity of CBA-CEACD3 to human CEA-expressing cells and CD3-bearing lymphocytes was determined by flow cytometry (Figures 3 and 4). When tested against CHO dhfr+ transfectoma cells expressing CEA, CBA-CEACD3 showed a significant increase in fluorescence intensity compared with background level obtained with control human IgG (Figure 3C). The fluorescence intensity of CBA-CEACD3 was comparable to that of its parental chimeric antibody Ch F11-39 against CEA (Figures 3B and C), whereas that of Ch OKT3 was almost identical to that of control human IgG (Figure 3A). CBA-CEACD3 also significantly bound to HPBMCs (Figure 4C) and its fluorescence intensity was very similar to that of the other

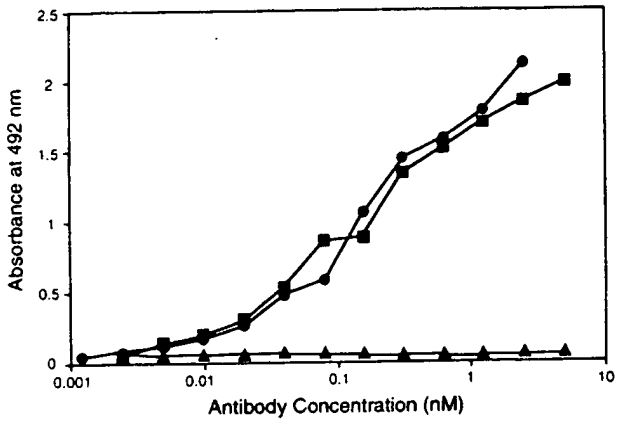

Figure 5. *Reactivity of CBA-CEACD3 with CEA in a SPEIA using antigen-coated plates (100 ng/100 µl/well). The plates were incubated with Ch OKT3 (▲), Ch F11-39 (■), or CBA-CEACD3 (●) for 1 hour at 37°C. After washing, the plates were successively incubated with a biotinylated goat anti-human IgG antibody (0.1 µg/ml), with horseradish peroxidase-avidin D (0.25 µg/ml), and then with 0.04% o-phenylene-diamine containing 0.006% H_2O_2. Values are presented as the mean of duplicate wells.*

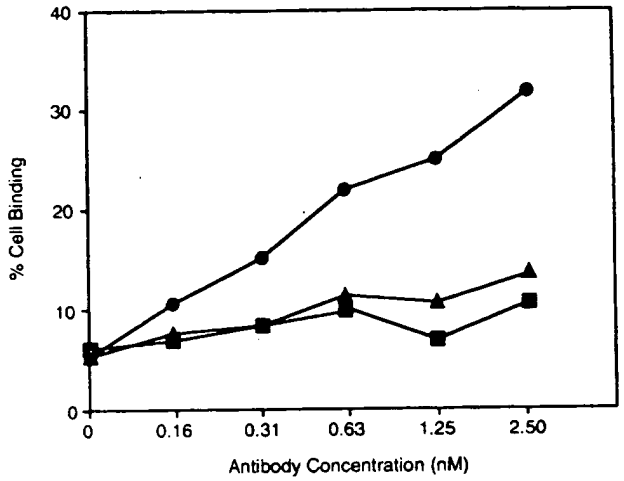

Figure 6. *Antibody-mediated binding of CEA-expressing cells to CD3-bearing cells. After fixation of HPBMCs on 96-well plates, CHO dhfr+ transfectoma cells expressing CEA were incubated in the plates with Ch OKT3 (▲), Ch F11-39 (■), or CBA-CEACD3 (●) for 2 hours 37°C. After washing, specifically bound cells were detected with MTT. Values are presented as mean of duplicate wells.*

parental chimeric antibody Ch OKT3 against CD3 (Figure 4A). The fluorescence intensity of Ch F11-39 against HPBMCs was also similar to that of control human IgG (Figure 4B). In case of control human IgG, the second small peak was seen in the middle range of fluorescence intensity (Figure 4, thin lines), intimating natural killer cells with Fc receptors and/or immature B cells with cell surface immunoglobulins.

Reactivity of CBA-CEACD3 with purified CEA. Binding reactivity of CBA-CEACD3 with CEA was also tested against purified CEA immobilized on 96-well plates. As shown in Figure 5, CBA-CEACD3 showed a binding curve almost identical to that of Ch F11-39, whereas no significant binding was observed with Ch OKT3.

Bifunctional binding activity of CBA-CEACD3 by a cell to cell binding assay. When the bifunctional binding activity of CBA-CEACD3 was tested by a cell to cell adhesion assay, the binding of CHO dhfr+ transfectoma cells expressing CEA to HPBMCs fixed on 96-well plates increased dose-dependently in the presence of CBA-CEACD3, whereas no significant binding was observed in the presence of Ch F11-39 or Ch OKT3 (Figure 6).

Tumor cell killing by CBA-CEACD3. The efficacy of CBA-CEACD3 in directing the killing of tumor cells expressing CEA in the presence of HPBMCs was also determined by a colorimetric assay using MTT (Figure 7). Lymphocytes with Ch F11-39 caused significant amounts of cytotoxicity. The level of cytotoxicity of lymphocytes with CBA-CEACD3 was markedly higher than that of lymphocytes with Ch F11-39, especially at lower E/T ratios. Ch OKT3 failed to exhibit antitumor activity. To demonstrate that the killing process by CBA-CEACD3 included the activity of cytotoxic T lymphocytes bearing CD3 in HPBMCs, an inhibition experiment was performed. As shown in Figure 7, the killing activity of CBA-CEACD3 could be inhibited significantly by the $F(ab')_2$ fragments of the parental mouse MAb OKT3.

Discussion

Several bispecific monoclonal antibodies of murine origin have been tested in human clinical trials (33, 34). However, a major disadvantage in the application of murine MAbs has been the elicitation of an immune response after repeated administration into humans (13). Here, we generated a mouse/human-chimeric bispecific antibody with dual specificities for CEA and CD3 by cross-linking of Ch F11-39 to Ch OKT3 using SPDP. Although several chimeric bispecific antibodies with dual specificities have recently been reported (35-38), there has been no report on chimeric bispecific antibodies with dual specificities for CEA and CD3.

When fivefold to eightfold molar excesses of SPDP were used for cross-linking, large amounts of heteroconjugates were obtained. However, the bifunctional binding activity of the heteroconjugates were low (data not shown) so that a threefold molar excess of SPDP was used in this study. When tested with SDS-PAGE, the band of Ch OKT3 was slightly broader than that of Ch F11-39, suggesting its different degree of glycosylation at the possible N-glycosylation site in the variable region of the Ch OKT3 heavy chain [Arakawa,

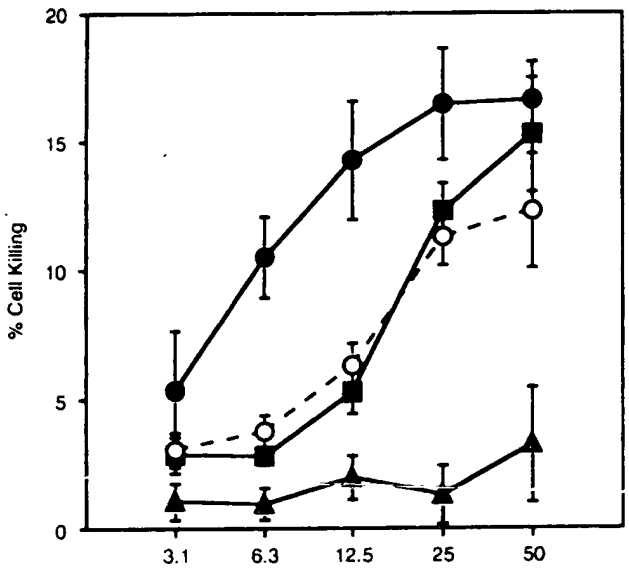

Figure 7. *Cytotoxicity of HPBMCs against CEA-expressing tumor cells in the presence of CBA-CEACD3 at a concentration of 5 nM. MKN-45 cells were attached to the bottoms of 96-well plates during 48 hours of incubation. HPBMCs at the indicated E/T ratios were added into each well of the plates in the presence of Ch OKT3 (▲), Ch F11-39 (■), or CBA-CEACD3 (●). The remaining living cells were detected using MTT. Values are presented as the mean ± SE of triplicate wells. A dotted line with open circles (○) shows the inhibition of CBA-CEACD3-mediated tumor killing of MKN-45 cells (5 nM CBA-CEACD3) by the F(ab')2 fragments of the parental mouse mAb OKT3 (100 nM).*

unpublished results]. This different degree of glycosylation may also give rise to a fairly broad band of CBA-CEACD3.

Flow cytometric analysis showed that CBA-CEACD3 can bind specifically to cells expressing CEA and to HPBMCs bearing CD3, respectively. When the flow cytometric profiles against HPBMCs were compared, the peak of fluorescence intensity of CBA-CEACD3 was similar to that of Ch OKT3 (Figures 4A and C). Against CHO dhfr+ transfectoma cells expressing CEA, very similar patterns were also seen between Ch F11-39 and CBA-CEACD3 (Figures 3B and C). In addition, when tested against purified CEA, CBA-CEACD3 showed a binding curve almost identical to that of Ch F11-39 (Figure 5). The binding of CBA-CEACD3 and Ch F11-39 to immobilized CEA were dose-dependently inhibited by another purified CEA preparation (data not shown). Since the numbers of moles of antibodies used were equalized, these results suggest that a threefold molar excess of SPDP might not decrease the original anti-CEA and anti-CD3 activities in CBA-CEACD3.

To see if the bifunctional binding activity of CBA-CEACD3 works at the same time, we developed a new cell to cell adhesion analysis, in which we could quantitatively determine the CEA-expressing cells specifically bound to HPBMCs fixed on 96-well plates by a colorimetric analysis based on the ability of live cells to enzymatically reduce MTT to a blue formazan product. The result obtained (Figure 6) revealed that CBA-CEACD3 is able to bind CEA-producing cells to CD3-expressing cells, indicating that both arms of CBA-CEACD3 are simultaneously working and can retarget T-cells to the tumor.

Furthermore, we determined the CEA-expressing tumor cell killing activity of CBA-CEACD3 in the presence of HPBMCs by a cytotoxicity assay using MKN-45 cells adhered to the well bottoms of 96-well plates followed by a colorimetric assay using MTT (Figure 7). The significant levels of cytotoxicity obtained with Ch F11-39 may be a result of antibody-dependent cell-mediated cytotoxicity (ADCC) activity *via* Fc receptors of NK cells in HPBMCs. The level of cytotoxicity of CBA-CEACD3 with targeting lymphocytes significantly increased when compared to that of Ch F11-39 with lymphocytes, especially at lower E/T ratios, suggesting that the antitumor activity of retargeted lymphocytes with CBA-CEACD3 resulted not only from ADCC of NK cells bul also from activity of cytotoxic T lymphocytes bearing CD3 in HPBMCs. This result also seems very meaningful because high E/T ratios are rather unexpected in humans *in vivo*. This finding was confirmed by an inhibition experiment, in which the killing activity of CBA-CEACD3 could be inhibited effectively by the $F(ab')_2$ fragments of the parental mouse MAb OKT3 (Figure 7).

Recently, chimeric bispecific antibody $F(ab')_2$ fragments have often been used in preference to intact chimeric bispecific antibodies in retargeted cellular cytotoxicity (36), since the former fragments have some advantages, such as their ability to easily penetrate into the tumor, the low risk of killing innocent bystander cells by binding to the Fc region of the antibody, and slower clearance from circulation. Such points should be borne in mind when chimeric bispecific antibodies are used in *in vivo* studies.

The results present here demonstrate that CBA-CEACD3 can be used *in vitro* to effectively retarget the killing of CEA-expressing tumor cells by HPBMCs.

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